CORYNEBACTERIA AS AN IMPORTANT GROUP OF SOIL MICROORGANISMS.

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The genus *Corynebacterium* Lehmann and Neumann has been studied diligently by medical bacteriologists, who naturally have devoted most of their energy to the study of the numerous pathologically important organisms of this genus, and very little attention has been paid to its purely saprophytic members. Harris and Wade (1915) seem to have been the first to conduct a search for corynebacteria occurring outside the human and animal body. They found "diphtheroids" of frequent occurrence, not only in various minor skin lesions, but also in the air, and concluded as a result of their study that "diphtheroids constitute a broader field of saprophytism than is generally appreciated". Kisskalt and Berend (1918) considered that several non-spore-forming bacteria, e.g., *Bact. helvolum* and *Bact. erythrogenes*, should really be included in *Corynebacterium* because of their characteristic mode of cell division. This classification has been adopted in the most recent edition of the manual of Lehmann and Neumann (1927), who also isolated such organisms from air and water.

The main points in the definition of *Corynebacterium* are: non-motile bacteria without endospore formation, gram-positive, not acid-fast, generally rod-shaped, but with a marked tendency to formation of irregular, club- or wedge-shaped, sometimes branching cells of a more varying size and shape than is usually found among the *Eubacteriales*, and multiplying by a characteristic "snapping" division of the cells, which causes the bacteria in microscopical preparations to appear in V- or III-like arrangements, or irregular groups sometimes compared to Chinese letters.

In a number of microbiological analyses of Australian soils the writer became aware of a remarkably frequent occurrence of bacterial colonies of this type on agar plates. An estimate of their relative frequency was carried out in the following manner: platings were made from soils in adequate dilutions (1:100,000 – 1,000,000) on 4–5 parallel plates of dextrose-casein-agar*; after incubation for 8 days at 28–30° C., all bacterial colonies were counted, and 40–50 colonies were picked at random and examined microscopically in nigrosin smears, by which method of preparation the characteristic cell shape and angular arrangement of the corynebacteria appears very clearly. The percentage of corynebacterium-like colonies was then calculated. Colonies of "doubtful" character, as were sometimes met with, were not reckoned as corynebacteria. From each soil a number of strains (generally 3–4) were isolated from typical colonies and studied in pure culture; they all conformed with the above definition of *Corynebacterium*. A closer description of the morphology and biology of these organisms is reserved for a later occasion.

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* Dextrose 2·0 gm.; casein, dissolved in 10 c.c. 0·1n NaOH, 0·2 gm.; K_2HPO_4 0·5 gm.; $MgSO_4$ 0·2 gm.; agar 15·0 gm.; water, 1,000 c.c.; pH 6·5–6·6.

TABLE 1.

Occurrence of Corynebacteria in Various Soils.

Soil No.	Character of Soil.	pH of Soil.	Bacteria.* Millions per gm.	Corynebacteria.	
				% of total Bacteria.	Millions per gm.
1*a*	Heavy loam, rich in organic matter, grass land, Sydney University.	4·8	14·4	29	4·2
1*b*	Same with addition of 1% cellulose and 0·1% ammonium sulphate, kept moist 5 months, room tpt.	4·2	0·6	8	0·05
1*c*	Same as 1*a*, with addition of 2% calcium carbonate, kept moist 14 days.	7·4	99·0	29	28·7
2*a*	Very light sand, poor in organic matter, vacant grass land, Bellevue Hill, Sydney.	5·1	8·0	37	3·0
2*b*	Same with addition of 1% calcium carbonate, kept moist 15 days, room tpt.	7·1	10·0	48	4·8
3	Coarse sand, poor in organic matter, Bathurst, N.S.W.	5·1	6·1	26	1·6
4	Light sand, rich in organic matter, Cooper Park, Sydney.	5·3	17·3	13	2·3
5	Heavy loam, rich in organic matter, from flower bed, Sydney University.	5·6	26·3	35	9·2
6	Light loam, very rich in organic matter, from Scone, N.S.W.	6·0	17·9	39	7·1
7	Light sand, poor in organic matter, Bellevue Hill, Sydney.	6·1	10·0	33	3·3
8	Red loam, poor in organic matter, Griffith, N.S.W.	6·1	18·2	64	11·6
9	Heavy loam, rich in organic matter, from pot experiments, Sydney University.	6·6	25·0	39	9·8
10	Loam, rich in organic matter, North Sydney.	6·9	20·2	60	12·1
11	Clay, rich in organic matter, sheep pen, McMaster Laboratory, Sydney University.	7·2	22·4	45	10·1
12	Loam, rich in organic matter, flower bed, Sydney University.	7·3	25·3	65	16·4

The figures in Table 1 show plainly that the corynebacteria account for a quite considerable part of the flora appearing on the plates, in some instances (soils 8 and 12) even up to two-thirds of the total number of bacterial colonies. There is a certain indication, although not very marked, that the percentage of corynebacteria increases somewhat with decreasing soil acidity. In the very acid soil, No. 1*b*, their frequency is remarkably low; this agrees with the fact that a degree of acidity corresponding to pH 4·3–4·6 appeared critical for most strains in pure culture.

Comparatively little work has yet been carried out on those non-spore-forming soil bacteria which do not distinguish themselves by striking physiological functions, such as nitrification, nitrogen fixation, denitrification, cellulose decomposition, etc. One of the few bacteriologists who have made a real study of the "ordinary" soil bacteria, viz., Conn (1925, 1928), describes the bulk of these as "slow-growers" or "punctiform-colony-forming bacteria", characterized by a poor

*Not including actinomycetes.

growth and uncharacteristic fermentation reactions in liquid media. Conn's groups III and IV seem to have something in common with the corynebacteria, but they have not yet been described in detail and were stated to be comparatively rare. The "slow-grower" which Conn found of most common occurrence—*Bact. globiformis*—was not met with in the present investigation, and upon the whole the corynebacteria dealt with here show little resemblance to Conn's "slow-growers", since they all grew well in broth as well as on agar media, and gave characteristic fermentative reactions. Most of their colonies on dextrose-casein-agar were of a fair size, from 0·5 to 2 mm. in diameter after 7–8 days, white to yellow, opaque, with a well-defined, even outline, of a mostly soft-pasty, sometimes slimy consistence; surface colonies were usually round, smooth and convex, deep colonies lens-shaped or ellipsoid. Probably we must here seek the identity of those soil bacteria which Greig-Smith (1911) interpreted as *Rhizobia*—nodule bacteria of leguminous plants. The work of later investigators has not revealed such large numbers of rhizobia in soil, and the nitrogen fixation recorded by Greig-Smith cannot be regarded as proving the organisms to be rhizobia.* On the other hand, Greig-Smith's description of the appearance of the colonies and the organisms in them agrees remarkably well with the corynebacteria in question. It is well known that nodule bacteria at a certain stage of their life cycle produce irregular, swollen and partly branched forms which may resemble corynebacteria, and this suggests that Greig-Smith may have mistaken such organisms for rhizobia. In order to test this point, platings were carried out from a few of the soils in Table 1 on the levulose-asparagine-citrate-agar employed by Greig-Smith, and the frequency of colonies of corynebacteria was estimated as before. Numerous corynebacteria were found to develop on this medium, as shown below:

Soil No.	Dilution.	Bacterial colonies, average per plate.	Percentage of corynebact.:	
			Levulose agar.	Casein agar.
2*b*	1 : 25,000	11·8	15	48
9	1 : 50,000	18·3	24	39
12	1 : 100,000	22·0	50	65

As a further test, 9 strains of corynebacteria known to be able to utilize asparagine were grown on slopes of Greig-Smith's agar, where they all, after 3 days at 28° C., produced a fair to abundant growth, convex, smooth, glistening, opaque and whitish, not unlike that of nodule bacteria on some favourable agar medium. Microscopical examination showed the presence of all the cell types which Greig-Smith describes thus: ". . . irregular outline and structure suggesting a sausage-skin stuffed more or less with marbles; and although the γ and Y forms were rare, the exclamation mark (!), the irregularly divided rod, and the club-

* Critical studies by several recent investigators have shown conclusively that nodule bacteria do not fix elementary nitrogen when grown outside the host plant, but several sources of error may give rise to a spurious nitrogen fixation.

shaped form were quite numerous." All this renders it very likely that Greig-Smith's "rhizobia" were really corynebacteria. That the present organisms should be rhizobia is out of question; none of them shows anything like the characteristic life cycle which distinguishes the nodule bacteria (Bewley and Hutchinson, 1920).

Some experiments were carried out in order to get an idea of the possible importance of the corynebacteria in the decomposition processes in the soil. 200-gm. portions of the soils 2*b* and 9 in Table 1, with 10 and 25 per cent. of water, respectively, received the following additions of organic matter:

0·5% dried and ground mycelium of *Penicillium* sp. (*"glaucum"*).
0·5% dried and ground leaves and stems of white clover.
1·0% dried and ground oats straw.

TABLE 2.

Multiplication of Corynebacteria during Decomposition of Organic Matter.

Soil and Addition.	Time.	Bacteria, Millions per gm.	Corynebacteria.		Actinomycetes, Millions per gm.
			% of Bacteria.	Millions per gm.	
Sand soil 2*b*, with 0·5% mycelium of *Penicillium* sp.	Start	10	48	4·8	3·2
	4 d.	1,333	13	173·3	(0)
	8 d.	550	13	71·5	0·5
	14 d.	237	38	89·1	1·5
	22 d.	63	35	22·1	1·2
Sand soil 2*b*, with 0·5% dry matter of white clover.	Start	10	48	4·8	3·2
	4 d.	765	27	206·5	0·3
	8 d.	469	19	89·2	0·8
	14 d.	170	23	39·1	1·2
	22 d.	59	26	15·3	1·8
Sand soil 2*b*, with 1·0% oats straw.	Start	10	48	4·8	3·2
	4 d.	95	35	33·3	0·9
	8 d.	86	32	27·5	1·3
	14 d.	97	33	32·1	1·2
	22 d.	41	30	12·3	0·8
Loam soil 9, with 0·5% mycelium of *Penicillium* sp.	Start	31	38	11·6	1·1
	4 d.	352	12	42·2	4·0
	8 d.	328	13	42·7	4·3
	15 d.	212	27	57·3	4·5
	22 d.	135	28	37·9	5·8
Loam soil 9, with 0·5% dry matter of white clover.	Start	31	38	11·6	1·1
	4 d.	264	25	66·0	2·0
	8 d.	272	25	68·0	3·8
	15 d.	194	28	54·4	5·3
	22 d.	139	20	27·8	3·7
Loam soil 9, with 1·0% oats straw.	Start	31	38	11·6	1·1
	4 d.	494	17	84·0	2·8
	8 d.	345	29	100·0	4·3
	15 d.	235	35	82·4	4·8
	22 d.	210	32	67·1	2·4

The soils were kept in large Petri dishes for 22 days at room temperature (18–20° C.). During this period, plate counts of bacteria were carried out four times, and the frequency of corynebacteria was estimated. The results, reproduced in Table 2, show that not only do the corynebacteria occur as a constituent of the soil flora, but they also multiply strongly during the decomposition of organic matter and thus presumably take a part in these processes. Their percentage is generally highest during the later stages of decomposition, and higher in soils with straw and clover than in soils with fungous mycelium, where a motile, yellow bacterium, probably *Bact. herbicola* or a related form, flourished abundantly, especially after 4–8 days.

Summary.

Bacteria possessing the characters of the genus *Corynebacterium* were found to occur as a numerically important group of microorganisms in Australian soils, accounting for 8 to 65 per cent. of the numbers of bacterial colonies developing on plates of dextrose-casein-agar. They appear to be active in the decomposition of organic matter in soil, particularly in the later stages of the process. They are probably identical with certain organisms previously recorded as rhizobia.

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